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Impact of Trichloroethylene and Toluene on Nitrogen Cycling in Soil

MARK E. FULLER[1,2]† AND KATE M. SCOW[1]*

Department of Land, Air and Water Resources[1] and Ecotoxicology Program,[2] University of California, Davis, Davis, California 95616

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The effects of trichloroethylene (TCE) and toluene on soil nitrogen-cycling activities were examined. Ammonium oxidation potential (AOP) was reduced after incubation with as little as 1 μg of TCE ml^{-1}, and the effects were generally greater when toluene was present and increased with longer exposure. Arginine ammonification potential and denitrification enzyme activity were constant regardless of TCE concentration or the presence of toluene, while nitrite oxidation potential (NOP) exhibited variable sensitivity. KCl-extractable ammonium levels increased dramatically after exposure to 30 and 60 μg of TCE ml^{-1} in the presence of toluene, whereas gamma-irradiated or sodium azide-treated soil incubated with the same concentrations of TCE and toluene showed no increase. Alfalfa-amended soils showed similar decreases in AOP and increases in extractable ammonium during incubation with 60 μg of TCE ml^{-1} and 20 μg of toluene ml^{-1}, although most probable number estimates of the ammonium oxidizer population showed no difference between exposed and unexposed soil. AOP and extractable ammonium returned slowly to control levels after 28 days of incubation in the presence of TCE and toluene. Activity assays to which various TCE and toluene concentrations were added indicated that AOP and NOP were relatively more sensitive to these compounds than was arginine ammonification potential. These results indicate that the soil microbial populations responsible for nitrogen cycling exhibit different sensitivities to TCE and toluene and that they may be more susceptible to adverse effects than previously thought.

Trichloroethylene (TCE), one of the most widespread pollutants detected in groundwater (24), can be biodegraded by indigenous soil microorganisms which also degrade toluene (8). The effects of TCE and toluene concentrations on the dynamics of the microbial populations responsible for toluene degradation and TCE cometabolism in soil have also been examined (19), and the mechanisms by which TCE interacts with degradative enzymes have been published (3, 7, 9, 13, 23).

Although no adverse effects of TCE on culturable heterotroph populations were detected by Mu and Scow (19), very high concentrations of chlorinated aliphatics, including TCE, have been shown to adversely affect some soil enzymes (15). However, extensive studies of the ecotoxicology of TCE in soil, especially in regards to nutrient-cycling activities, have not been performed nor have the synergistic effects of TCE in combination with toluene been assessed.

This paper reports the effects of different TCE concentrations in the absence and presence of toluene on microbial processes involved in the soil nitrogen cycle. The effects of different concentrations of toluene alone on nitrogen-cycling activities in soil, and of TCE and toluene on the general soil community (bacteria, protozoa, and nematodes), are presented elsewhere (11, 12).

MATERIALS AND METHODS

Soil incubation. Yolo silt loam (Typic Xerorthents; 16% H_2O, 1.2% organic carbon [pH 6.8]) was passed through a sieve (mesh, 2 mm) and stored at 4°C until use. Prior to starting the experiments, soil was incubated at 25°C for 18 to 24 h. Fifty grams (dry weight) was weighed into 254-ml bottles equipped with 24-mm-diameter mininert caps (Dynatech Precision Sampling Corp., Baton Rouge, La.). Sufficient bottles were prepared so that triplicate bottles of each treatment at each time point could be sampled. Sterile soil was prepared by irradiation with 3.5 to 4.0 megarads from a ^{60}Co source. Sodium azide was used as a biostatic agent by treating soil with 100 mg of NaN_3 ml^{-1}. Biodegradation of TCE and toluene was assessed by measuring the disappearance of the chemicals from the headspace by using gas chromatography. Sorption and Henry's partition coefficients were used when calculating TCE and toluene additions; all concentrations are reported as micrograms $milligram^{-1}$ of soil solution (8). The oxygen concentration in each sealed bottle was measured with an S-3A O_2 analyzer (Applied Electrochemistry, Inc., Sunnyvale, Calif.) immediately before terminating each incubation. After incubation, TCE and toluene were removed from the soil by purging with moist air for 20 min, while shaking each bottle every 4 min. Gas chromatographic analysis showed that this purging reduced the highest TCE concentrations to below 1 μg ml^{-1} and reduced toluene concentrations to below 5 μg ml^{-1}. Further manipulation of the soil during assay preparation reduced residual TCE and toluene to below detection limits. The soil pH was measured in a 2:1 soil paste in distilled water.

To examine the effects of TCE and toluene on the in situ ammonification and mineralization of plant material, 10 g of soil was amended with alfalfa at a rate of 2 mg g^{-1} of soil before the addition of 60 μg of TCE ml^{-1} and 20 μg of toluene ml^{-1}. The alfalfa was air dried, ground, and passed through a 20-mesh sieve. The alfalfa had a carbon to nitrogen ratio of 18:2, as determined by the Division of Agriculture and Environmental Resources Analytical Laboratory, University of California, Davis, Davis, Calif.

The reversibility of the effects of exposure to high concentrations of TCE and toluene also were assessed. Soil was incubated for 28 days with 60 μg of TCE ml^{-1} and 20 μg of toluene ml^{-1}. After TCE and toluene were removed by venting, exposed and unexposed (control) soil was assayed for ammonium oxidation potential (AOP), nitrite oxidation potential (NOP), and KCl-extractable ammonium; the remaining bottles were incubated for an additional 7 and 30 days, at which time they also were assayed.

Activity assays. All assays performed, except that for denitrification enzyme activity (DEA), were described previously in detail (11). Arginine ammonification potential (AAP) was determined by measuring the amount of ammonium released after soil samples were shaken for 1 h in copolymer centrifuge tubes containing an arginine solution. The direct effects of TCE and toluene on AAP were examined by using Teflon Oak Ridge tubes sealed with 24-mm-diameter mininert caps. AOP was assayed by shaking soil samples for approximately 24 h in phosphate buffer containing ammonium sulfate and sodium chlorate and measuring the nitrite produced. Because this research was primarily concerned with the relative differences between treatments, and not with the absolute values of AOP, the conversion of the chlorate to chlorite over the course of the assay

* Corresponding author. Mailing address: Department of Land, Air and Water Resources, University of California, Davis, Davis, CA 95616. Phone: (916) 752-4632. Fax: (916) 752-1552. E-mail: kmscow@ucdavis.edu.

†Present address: Environmental Research Division, ER/203-J070, Argonne National Laboratory, Argonne, IL 60439.

was not determined and was assumed to be negligible. NOP was assayed by shaking the soil samples for approximately 24 h in phosphate buffer containing sodium nitrite and nitrapyrin and measuring the amount of nitrite consumed. Controls for each assay consisted of soil samples shaken in phosphate buffer only. Direct effects of TCE and/or toluene on AOP and NOP were assayed in bottles sealed with 24-mm-diameter mininert caps.

Nitrite concentrations were determined by the spectrophotometric method of Kartikeyan et al. (16). Two-milliliter samples from the AOP or NOP assays were centrifuged at 10,000 × *g* for 5 min to pellet soil particles. A 0.5-ml aliquot of the cleared supernatant was mixed with 0.2 ml of 0.012% rhodamine-6-G (Sigma Chemical Company, St. Louis, Mo.), 0.2 ml of 12.5 M H_2SO_4, and 1.1 ml of water, and the optical density at 525 mm was measured. This nitrite quantification method has been shown to be very comparable to more established methods when assaying foods for nitrite content (16) and is assumed to be reliable for cleared soil extracts as well.

Ammonia-oxidizing bacteria in soil were enumerated by a slight modification of the standard most probable number method of Schmidt (1982) and by using 24-well polystyrene microtiter plates.

DEA was determined by the acetylene block technique (21, 22). Five grams of soil was suspended in 10 ml of water containing 0.51 mg of sodium nitrate ml^{-1}, 1.8 mg of glucose ml^{-1}, and 0.125 mg of chloramphenicol ml^{-1}. The slurry was sparged for 5 min with nitrogen while being gently shaken, and the bottles were then capped and sealed with aluminum crimp seals. Ten percent of the headspace was replaced with commercial-grade acetylene, and the bottles were shaken (150 rpm) for 1 to 2 h, at which time headspace nitrous oxide levels were determined by gas chromatography.

Analytical techniques. TCE and toluene were quantified by gas chromatography as described previously (10). The concentration of nitrous oxide formed during the DEA assay was determined with a gas chromatograph (model 427; Packard Instrument Co., Downers Grove, Ill.) equipped with a stainless steel column (inside diameter, 31.7 mm; length, 3.66 m) packed with 100/120-mesh Chromsorb 106 porous polymer (Alltech Associates Inc., Deerfield, Ill.) and an electron capture detector. The column temperature was 120°C, and the detector temperature was 320°C. The carrier gas was 5% methane in argon.

Data analysis. Data were analyzed by analysis of variance using StatView (version 4.0, Abacus Concepts Inc., Berkeley, Calif.). Treatment means were tested for significant differences at the 5% level by the Bonferroni/Dunn post hoc procedure, although most differences were significant at *P* values of 0.001 or smaller.

RESULTS AND DISCUSSION

Biodegradation kinetics. The biodegradation of TCE and toluene in Yolo silt loam was highly dependent upon the TCE concentration (Fig. 1), confirming observations of a previous study (19). TCE at a concentration of 30 μg ml^{-1} retarded the degradation of toluene and inhibited its own degradation. Sixty micrograms of TCE ml^{-1} inhibited both toluene and TCE biodegradation, and no TCE was degraded in the absence of toluene. No significant differences in final oxygen concentrations or soil pH values between treatments were detected (data not shown).

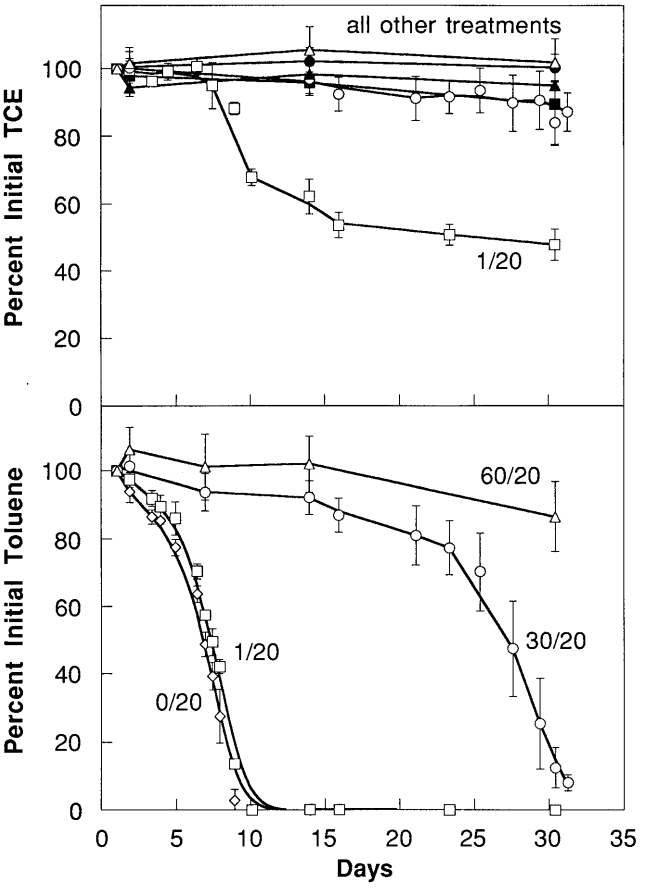

FIG. 1. Effects of concentrations of TCE and toluene on TCE and toluene biodegradation in Yolo soil. (A) TCE biodegradation. (B) Toluene biodegradation. Ratios used to label curves are micrograms of TCE ml^{-1}/micrograms of toluene ml^{-1}. Other ratios are represented by symbols. Error bars indicate standard deviations. (■), 1/0; (●), 30/0; (○), 30/20; (△), 60/0; (▼), 60/20.

Effects on ammonium oxidation. It was readily apparent that the oxidation of ammonium by indigenous soil microorganisms was the most TCE-sensitive process examined. The AOP of soil was strongly dependent upon the concentration of TCE to which it was exposed and on whether toluene was present. After 28 days of incubation, significant decreases in AOP were observed for all soils exposed to TCE (Fig. 2A). Effects were consistently greater when toluene was present, indicating synergistic interaction of the two compounds on the target process. When soil was amended with alfalfa and exposed to 60 μg of TCE ml^{-1} and 20 μg of toluene ml^{-1}, AOP was reduced to 15 and 30% of the control value after 7 and 37 days of incubation, respectively (Table 1). Absolute activity levels on day 37, however, were approximately fourfold higher than those observed previously in unamended soil. The number of ammonium oxidizers rose from 10^2 to 10^5 cells ml^{-1} between day 7 and day 37 of the incubation in both control and exposed soil. The effects on AOP of exposure to 60 μg of TCE ml^{-1} and 20 μg of toluene ml^{-1} for 28 days were observed to be reversible; soil AOP levels were not significantly different from control levels 30 days after TCE and toluene were removed from the system, although they were still depressed by 33% relative to the control (Fig. 3A). The most probable number of ammonium oxidizers after exposure was 10^5 per ml, and this number remained stable upon further incubation for 7 and 30 days after venting. Further experiments indicated that the inhibition of AOP by TCE may be due to direct effects of TCE, since the oxidation of ammonium in short-term activity assays (Fig. 4A) was greatly reduced even at levels of 1 μg ml^{-1}.

The primary mechanism by which these compounds inhibit ammonium oxidation is postulated to be the interaction of TCE and toluene with the ammonium monooxygenase (AMO) enzyme system. Nitrifying enrichment cultures derived from activated sludge were shown to have their ammonium consumption activity reduced by 50% in the presence of only 0.81 μg of TCE ml^{-1}, whereas 84 μg of toluene ml^{-1} was required to bring about the same degree of inhibition (4). Studies with pure cultures of the ammonium-oxidizing bacterium *Nitrosomonas europaea* have demonstrated that TCE (14, 20) and other chlorinated aliphatics and aromatics (17) act to inhibit AMO activity by competing with ammonium for the active site on ammonium monooxygenase. Rasche et al. (20) found that TCE could be cometabolized by *N. europaea* only at the cost of substantial inactivation of AMO. Furthermore, experiments by Hyman et al. (14) showed that *N. europaea* cells exposed to TCE exhibited major inhibition of AMO and recovery from exposure required de novo enzyme synthesis. In light of these

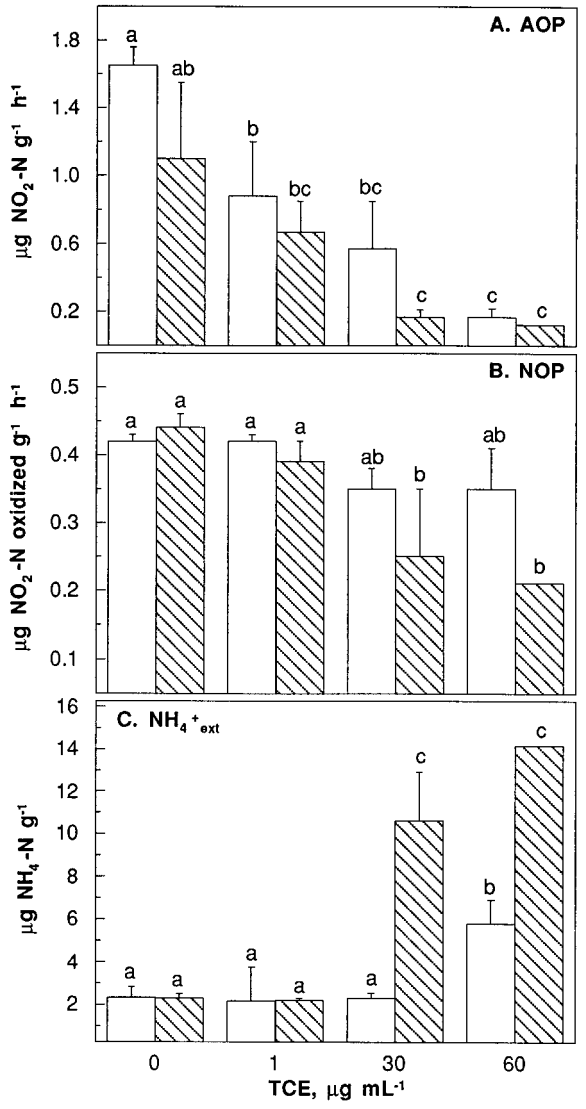

FIG. 2. Effects of different concentrations of TCE and the absence (□) or presence (▨) of toluene (20 μg ml^{-1}) on indicated nitrogen-cycling activities of soil after 28 days of incubation. Bars marked with different letters are significantly different ($P \leq 0.05$). Error bars indicate standard deviations.

mixed and pure culture results, it is likely that exposure of soil to TCE reduced AOP by a similar mechanism and ammonium-oxidizing organisms were not able to keep pace with the rate of deactivation. Ammonium oxidizers are believed to be obligate lithoautotrophs, and hence, they are wholly dependent on the oxidation of ammonium for the energy needed for growth and maintenance. Our soil incubation studies, however, failed to show a direct relationship between reduced soil AOP and the number of ammonium-oxidizing bacteria. It may be that the residual AMO activity was sufficient to maintain the ammonium-oxidizer population, or perhaps the AOP assay underestimated the in situ ammonium oxidation rate.

A synergistic effect on AOP of TCE and toluene was clearly seen in the soil incubation experiments (Fig. 2 and 3) but was not observed when TCE and toluene were added directly to the AOP assay to assess acute toxicity (Fig. 4A). Soil incubated for 28 days with TCE alone or with TCE and toluene exhibited dose-response patterns, showing both more inhibition of AOP

TABLE 1. Effects of 60 μg of TCE ml^{-1} and 20 μg of toluene ml^{-1} on the concentration of $NH_4^+{}_{ext}$ and AOP of alfalfa-amended soil[a]

Day	Treatment group	$NH_4^+{}_{ext}$ concn (μg of NH_4-N g^{-1})	AOP (μg of NO_2-N g^{-1} h^{-1})
0[b]	Control	0.10 (0.10) a	ND[c]
	Exptl	3.43 (0.19) b	ND
7	Control	0.28 (0.47) a	4.23 (1.03) a
	Exptl	13.71 (0.92) b	0.65 (0.18) b
37	Control	1.96 (0.14) A	2.86 (0.10) a
	Exptl	18.75 (3.87) B	0.87 (0.05) b

[a] Values followed by different letters are significantly different. ($P \leq 0.05$). Statistics were calculated only for the data from individual time points. Numbers in parentheses are standard deviations.
[b] Sample was taken 5 h after TCE and toluene were added to soil.
[c] ND, not determined.

with higher TCE concentrations and an additive effect of TCE and toluene. For acute toxicity, however, maximum inhibition (80%) of AOP by TCE alone was measured at 1 μg of TCE ml^{-1} (7.6 μM) and did not significantly increase with increasing TCE concentrations or the presence of 20 μg of toluene ml^{-1} (217 μM). The acute toxicity of TCE to AOP was much more pronounced than the chronic toxicity associated with 1 or 30 μg of TCE ml^{-1}, whereas the acute and chronic toxicities of 60 μg of TCE ml^{-1} to AOP were similar. These differences may be artifacts of how the exposure to TCE occurred, since chronic toxicity was assessed on ammonium-oxidizing organisms more or less undisturbed in the soil matrix and acute toxicity was determined in a shaken soil slurry. The longer duration of the soil incubation experiments also may have permitted an easing of inhibition due to TCE and toluene degradation or an acquired resistance to the chemicals by the ammonium-oxidizing population. It also should be noted that the AOP assay employed in this research did not allow us to distinguish between ammonium oxidation by AMO or by the methane monooxygenase of methanotrophs. Therefore, the conclusion that TCE and toluene disrupt ammonium oxidation in soil solely through the interaction of these compounds with AMO must be considered to be tentative until more detailed studies are conducted.

Effects on nitrite oxidation. Soil NOP was not as sensitive to TCE and toluene concentrations as was AOP. After 28 days of incubation, NOP activities were 55 and 43% of those of the control in soil exposed to 30 and 60 μg of TCE ml^{-1}, respectively, and 20 μg of toluene ml^{-1} (Fig. 2B). However, NOP measured during another experiment with a 28-day exposure to TCE and toluene was not different from control levels (Fig. 3B). Concentrations of 30 and 60 μg of TCE ml^{-1} alone, or with toluene, added to short-term assays significantly reduced NOP to levels of 80 and 45% of the control, respectively (Fig. 4B). The lowest levels of NOP were associated with the combination of 60 μg of TCE ml^{-1} and 20 μg of toluene ml^{-1}.

There has been little published research on the impacts of organic pollutants on NOP, probably as a result of widespread acceptance that ammonium oxidation is the most sensitive soil process. This research, however, showed that TCE and toluene did adversely affect nitrite oxidation and demonstrated synergistic interactions. Reductions in NOP could represent direct interactions of TCE and toluene with the nitrite-oxidizing machinery, a decrease in nitrite-oxidizing populations due to the dwindling amount of nitrite being produced by the ammonium oxidizers, or a combination of the two. Nitrite is oxidized by a somewhat nonspecific nitrite oxidoreductase (6), leaving open the possibility that TCE could interact directly with the enzyme

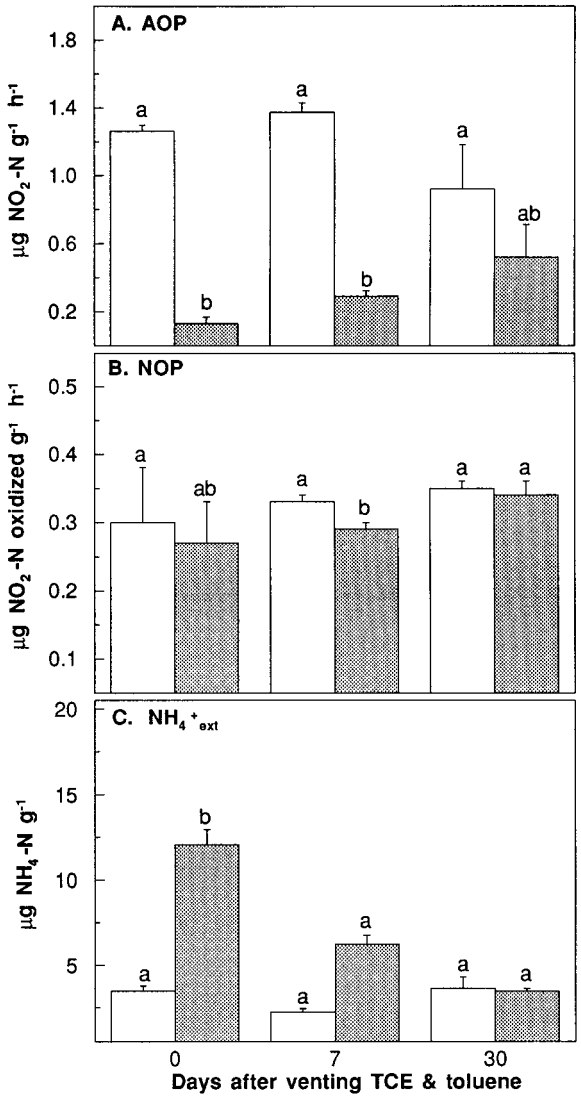

FIG. 3. Nitrogen-cycling activities of control soil (□) and soil exposed to 60 μg of TCE ml^{-1} and 20 μg of toluene ml^{-1} (▨) after 28 days of incubation, venting, and further incubation for 7 or 30 days. Bars marked with different letters are significantly different ($P \leq 0.05$). Statistics were not calculated for data across time points. Error bars indicate standard deviations.

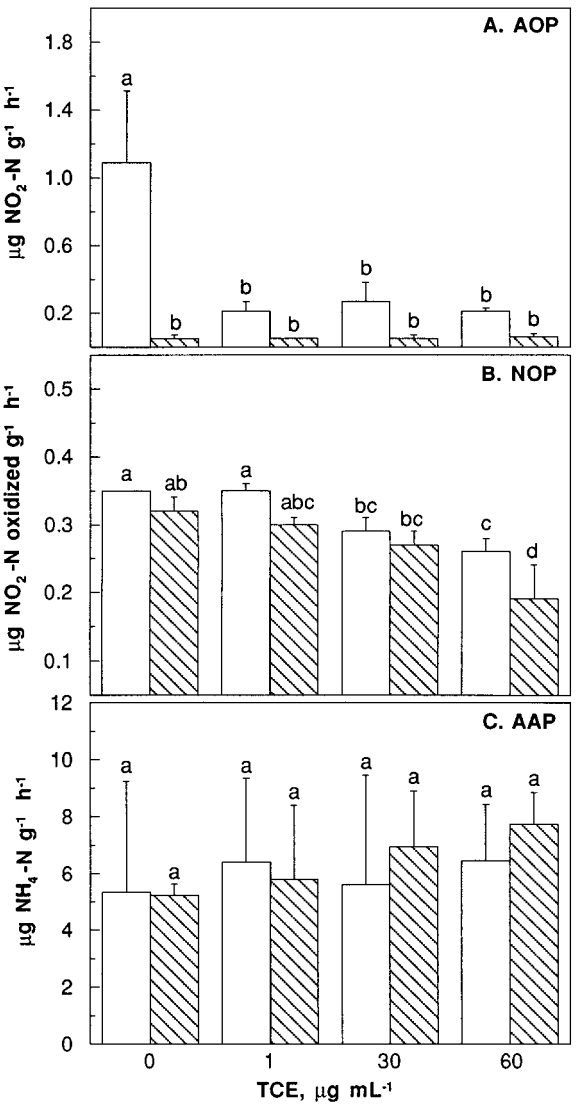

FIG. 4. Direct effects of different concentrations of TCE in the absence (□) or presence (▨) of toluene (20 μg ml^{-1}) on nitrogen-cycling activities. Bars marked with different letters are significantly different ($P \leq 0.05$). Error bars indicate standard deviations.

to inhibit NOP. Nitrite oxidizers are generally believed to be lithoautotrophs, although members of the genus *Nitrobacter* have been observed to grow heterotrophically, with each species exhibiting differing degrees of mixotrophy and diauxy on nitrite and simple organic compounds (5). Possessing heterotrophic capabilities may help nitrite oxidizers maintain viability when nitrite oxidation is inhibited or when there is insufficient nitrite available and may help explain why soil NOP was less sensitive to TCE and toluene than AOP.

Effects on ammonification. TCE exposure, in the presence or absence of toluene, did not affect nitrogen mineralization, as estimated by AAP (Table 2). However, soil exposed to 30 or 60 μg of TCE ml^{-1} possessed significantly more KCl-extractable ammonium ($NH_4^+{}_{ext}$) (Fig. 2C), and levels were higher when toluene was present. When soil was amended with alfalfa and exposed to 60 μg of TCE ml^{-1} and 20 μg of toluene ml^{-1}, $NH_4^+{}_{ext}$ levels rose to almost 10 times those observed in unexposed soil (Table 1). The concentration of $NH_4^+{}_{ext}$ took 30 days to return to control levels after incubation with 60 μg of TCE ml^{-1} and 20 μg of toluene ml^{-1} (Fig. 3C). Neither TCE alone nor TCE and toluene reduced AAP when added directly to activity assays (Fig. 4C), nor were there differences in $NH_4^+{}_{ext}$ concentrations (data not shown). The increase in the level of $NH_4^+{}_{ext}$ after long-term incubation with TCE and toluene was shown to be biologically mediated, since gamma-irradiated and sodium azide-treated soil showed no differences in the levels of $NH_4^+{}_{ext}$ after a 28-day incubation with 1, 30, or 60 μg of TCE ml^{-1} and 20 μg of toluene ml^{-1} (data not shown).

Since many organisms are believed to have the ability to ammonify nitrogenous substances (18), any activities lost by organisms affected by TCE would presumably be compensated for by the activity of less sensitive species. AAP has also been used a general measure of soil microbial activity (1, 2), and as such, the results of this research confirm work presented else-

TABLE 2. Effects of TCE and/or toluene (20 μg ml^{-1}) concentrations on soil AAP and DEA[a]

TCE (μg ml^{-1})	Toluene	AAP (μg of NH_4-N g^{-1} h^{-1})	DEA (ng of N_2O-N g^{-1} h^{-1})
0	−	3.62 (0.51) a	713 (336) a
	+	3.87 (0.27) a	557 (313) a
1	−	4.15 (1.58) a	500 (290) a
	+	4.10 (0.22) a	651 (381) a
30	−	4.07 (0.24) a	900 (305) a
	+	2.53 (0.47) a	627 (297) a
60	−	4.70 (1.13) a	432 (380) a
	+	2.71 (ND[b]) a	488 (254) a

[a] Values followed by different letters are significantly different ($P \leq 0.05$). Numbers in parentheses are standard deviations.

[b] ND, not determined. Standard deviation was not determined due to leakage of TCE and toluene from one replicate.

where that the overall microbial activity of soil is not affected by these concentrations of TCE (12). The continued accumulation and persistence of high $NH_4^+{}_{ext}$ levels (up to 95 μg of NH_4-N ml^{-1} or 5 mM) is one way in which TCE may indirectly impact the soil ecosystem. The possible consequences of these increases are explored elsewhere (11).

Effects on denitrification. The concentrations of TCE and toluene tested had no observable effects on soil denitrification as measured by DEA (Table 2). Although soil exposed to 60 μg of TCE ml^{-1} and 20 μg of toluene ml^{-1} exhibited somewhat lower denitrification activities than did most of the other treatments, the differences were not statistically significant due to the high inherent variability of this assay. The biochemical mechanism for denitrification is based on sequential reductions of nitrate, with the final product being either nitrous oxide or molecular nitrogen, and therefore no direct biochemical mechanism for TCE inhibition of denitrification is readily apparent.

In addition to what already is known about the sensitivity of the populations that degrade TCE and toluene to these two chemicals (19), our work has established that ammonium oxidation, and to lesser extent, nitrite oxidation, is reduced in the presence of TCE and toluene, resulting in a buildup of ammonium. TCE was also shown to act synergistically with toluene to inhibit both ammonium and nitrite oxidation. It is expected that these chemicals (and closely related compounds) could disrupt soil nitrogen cycling even at moderately low concentrations. One area that needs to be more fully explored is the interaction of mixtures of environmental pollutants with AMO, with particular emphasis on the differences in sensitivities between indigenous and laboratory strains of ammonium oxidizers. From an ecological restoration standpoint, having insights into these differences may aid in the assessment of both the present state of contaminated soils and the degree of recovery of soils undergoing remediation.

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